Phenotyping Milk Proteins: A Review

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Abstract

A variety of methods for phenotyping whey proteins and the caseins is summarized. The writer suggests what he believes are the best methods for phenotyping genetic variants of the milk protein system depending upon the availability of apparatus. No single method has yet been devised to simultaneously type the variants of α_{s1}- and β-caseins.

Historical

The classical studies of Aschaffenburg and Drewry (9) concerning genetic polymorphism of β-lactoglobulin were, without a doubt, the impetus to nearly 15 years of intensive research on milk protein variation. Using paper electrophoresis they determined that β-lactoglobulin (β-Lg) exists in two forms termed β-A and B, which can occur either singly or in pairs. Shortly after this discovery, Blumberg and Tombs (14) reported genetic variation in the second most abundant whey protein, α-lactalbumin (α-La); two forms, A and B, exist.

The first evidence of variations in the caseins was again a contribution of Aschaffenburg (3) who, studying the milks of individual cows by paper electrophoresis with urea, noted that β-casein (β-Cn) exists in three forms, which he termed β-Cn A, B, and C. Aschaffenburg's observations were confirmed by Thompson et al. (29), whose research was concerned with polymorphism of β-caseins of American cattle. They employed starch-gel and polyacrylamide-gel electrophoresis at alkaline pH. In 1962 Thompson et al. (28) showed that α_s-casein (now termed α_{s1}-Cn), like β-casein, is polymorphic, with three forms of the protein (A, B, and C) found in the United States (19). A fourth variant, D, has since been reported (17).

Almost simultaneously, but independently, Neelin (22), Woychik (34), and Schmidt (24) reported genetic variation in κ-casein (κ-Cn). Finally, El-Negoumy (16) and Groves (18) disclosed that γ-casein (γ-Cn) is genetically variable, as are a number of other minor casein components related to γ-Cn and β-Cn. Groves (18) has recently reviewed this subject. The current status of milk protein variations has been thoroughly reviewed by Aschaffenburg (8).

Interestingly, all of the aforementioned contributions have been advanced by researchers using a variety of zonal electrophoretic methods including paper, starch-gel, agar-gel, and polyacrylamide-gel electrophoresis and variations thereof. Therefore, selection of any one method depends on the protein variant to be detected, resolution of components desired, and availability of equipment. No single method is capable of detecting all polymorphs simultaneously; for example, β-caseins A^1, A^2, and A^3 can only be determined by the acid-gel electrophoretic method of Peterson and Kopfler (23), not by conventional alkaline pH methods. It is the purpose of this review to suggest those methods

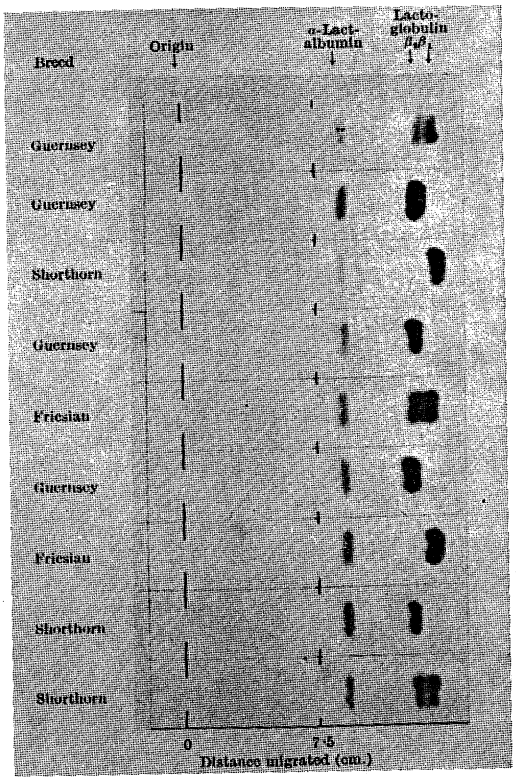

FIG. 1. β-Lactoglobulins in the milk of nine individual cows (bromophenol blue staining). β_1 and β_2 are now referred to as β-A and β-B. Reproduced with permission from Nature, 176: 218. 1955. (Fig. 1.)

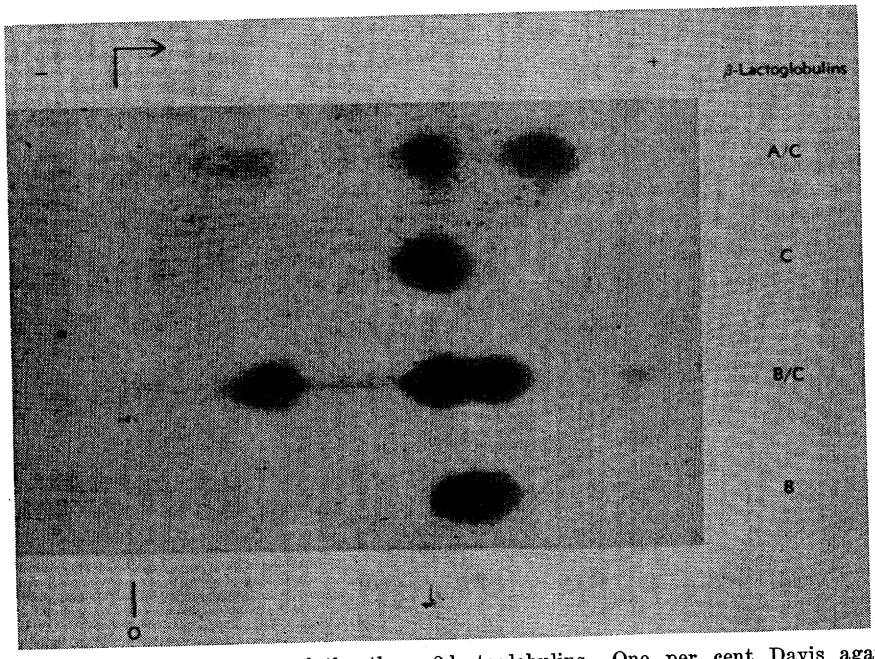

FIG. 2. Agar-gel electrophoresis of the three β-lactoglobulins. One per cent Davis agar; 0.0165 M-veronal, pH 8.6; 10 v/cm for 90 min; 10 mg protein/ml. O = Origin. Reproduced with permission from J. Dairy Sci., 48: 128. 1965. (Fig. 1.)

which the writer has observed to be acceptable and convenient for milk protein phenotyping, and to emphasize their limitations.

Whey Proteins

β-Lactoglobulins and α-lactalbumins. Figure 1 illustrates the separation of β-lactoglobulins (β-Lg) A and B by paper electrophoresis at pH 8.6, veronal buffer, $\Gamma/2 = 0.05$ (9). The two variants are clearly resolved; the disadvantage of this method is that β-Lg C and D would be observed with difficulty, if at all. Presumably, polyacrylamide-gel electrophoresis (PAE) should be far superior to paper electrophoresis for the resolutions of the C and D variants from the B variant. However, it has been the experience of this writer that PAE, while quite acceptable for the resolution of AB, still leaves much to be desired for the resolution of C or D from B.

Several alternative methods are available. First, perform agar-gel electrophoresis (6) at pH 8.6 in veronal buffer (Fig. 2) where β-Lg A, B, C, and presumably D are clearly resolved. The advantage of this method, aside from excellent resolution, is the ease with which the operation is performed—one need only have glass plates for support of the agar, a simple cell, and a power supply.

Another useful method described by Grosclaude et al. (17) utilizes the earlier starch-gel (SGE) method of Wake and Baldwin (33) with the incorporation of mercaptoethanol in the gel. However, while β-Lg A and B or A and D

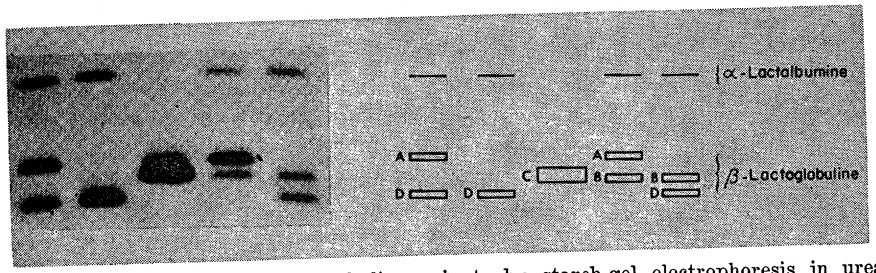

FIG. 3. Relative mobility of β-lactoglobulin variants by starch-gel electrophoresis in urea [Wake and Baldwin (33)] and mercaptoethanol. Reproduced with permission from Ann. Biol. Anim., Bio-Chem. Biophys., 6: 215. 1966. (Fig. 2.)

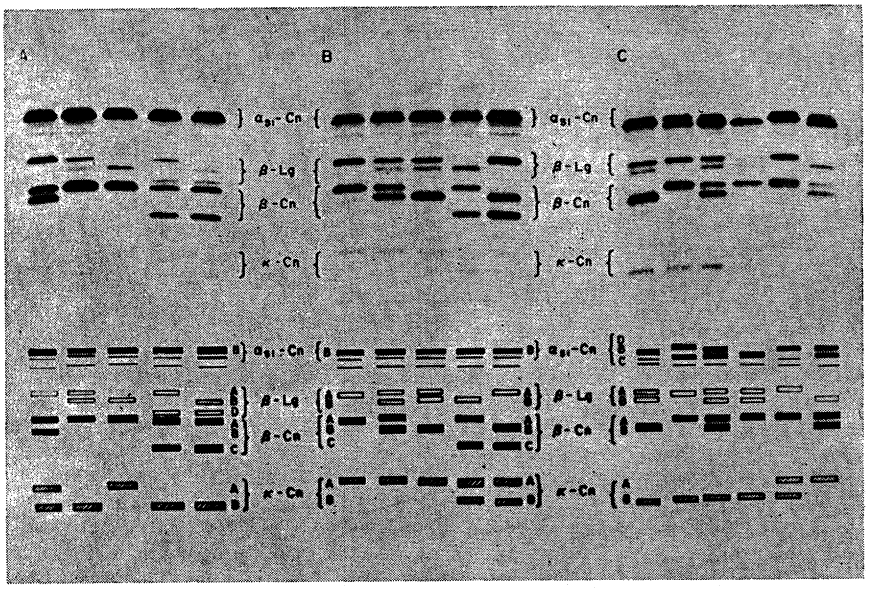

FIG. 4. Starch-gel electrophoresis of whole milk samples in urea and mercaptoethanol (21). Letters on bottom half of photograph refer to genetic types of the milk proteins. (Photograph courtesy Dr. W. Michalak, Warsaw, Poland.)

are clearly resolved, β-Lg B and C cannot be differentiated (Fig. 3). The earlier starch-gel method of Bell (13) resolved all three lactoglobulins (A, B, and C) without urea or mercaptoethanol. With regard to starch-gel electrophoresis, this writer can find no better example of general phenotyping of milk proteins than that illustrated by Figure 4. Lactoglobulins A, B, and D are clearly resolved in this urea-mercaptoethanol-containing system and little doubt exists that β-Lg C would also be resolved. Aschaffenburg and Thymann (12) had earlier developed an SGE method for β-Lg phenotyping which failed to differentiate clearly between the B and C variants. Arave (2) has demonstrated the usefulness of gel electrophoresis at low pH for phenotyping β-Lg A and B. Arave reported, however, that since a sample of β-Lg C was unavailable, it was not known if this variant could be resolved by electrophoresis at low pH.

Polyacrylamide-gel electrophoresis has also been used for the phenotyping of β-lactoglobulin by Aschaffenburg (5) at pH 8.6. While the resolution of the AB variants is good, it has been the experience of Kiddy (unpublished) and the writer that the method leaves much to be desired for resolution of the C variant. The recommended procedures for the phenotyping of β-Lg are either agar-gel electrophoresis (Fig. 2) or starch-gel electrophoresis (Fig. 4). If one assumes that β-Lg C and D are absent, any of

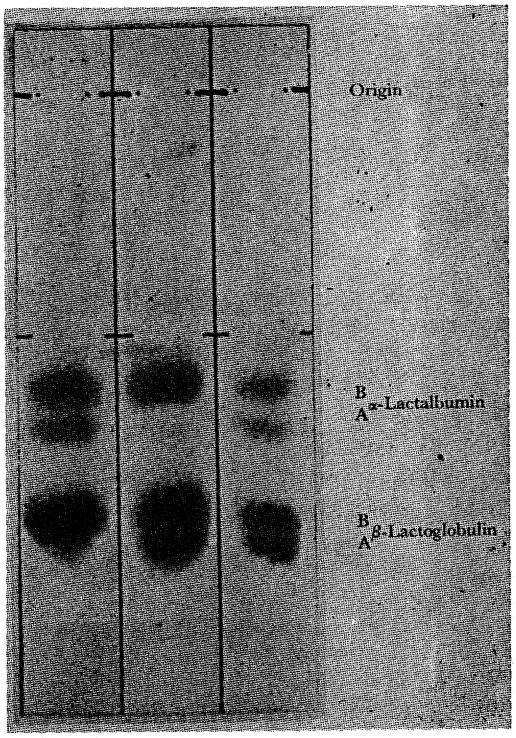

FIG. 5. Examples of α-lactalbumin and β-lactoglobulin polymorphism in Boran cattle (Kenya). Paper electrophoresis of milk filtrate at pH 8.6. Reproduced with permission from Man and Cattle. 1963. Occasional paper no. 18, Royal Anthropological Institute, p. 50. (Plate VI.)

the listed methods would be satisfactory for the resolution of A and B.

All that has been stated for β-lactoglobulins can be restated for α-lactalbumin except that only two variants of this protein have been reported, A and B, of which A occurs only in *Bos indicus* cattle. The B variant occurs in the milks of *Bos taurus* (4,6). Figure 5 illustrates their resolution by paper electrophoresis at pH 8.6. One must be cautioned, however, that the SGE method of Michalak (21) (Fig. 4 and 11) is useless for α-La phenotyping since it migrates in the α_{s1}-casein region.

Caseins

One of the factors which contributed to the impetus in studying genetic polymorphism was the now classical use of zonal electrophoresis (starch-gel in urea) for the identification of casein components. It is the principle of this method which facilitated many interesting discoveries concerning the caseins. However, the first concrete illustration of casein polymorphism was once again the contribution of Aschaffenburg (3) who, using paper electrophoresis at pH 7.15, 6.0 M urea (Fig. 6), illustrated polymorphism of β-casein in whole caseins. While the method is not applicable to phenotyping α_{s1}- or κ-casein, it stood as the impetus to several years of concentrated research on milk protein polymorphism.

β-Caseins have been conveniently phenotyped as A, B, and C by paper electrophoresis, starch-gel (29), and polyacrylamide-gel electrophoresis (29). All three methods at alkaline pH's are clearly capable of this differentiation. However, Peterson and Kopfler (23) have shown by gel electrophoresis (polyacrylamide) at pH 2.8 that β-casein A is subdivided into at least three distinct genetic forms which they refer to as β-A^1, A^2, and A^3, an important discovery confirmed by many others (7, 11). Thus, while alkaline-gel electrophoresis is suitable for phenotyping the B, C, and D (11) variants and κ-caseins A and B (34), it is of no value in resolving the β-A components. However, at pH 2.8 (Fig. 7) the resolution of the A components is striking. That the D variant cannot be resolved from the B variant is not a deterrent to the usefulness of acid gels since the D variant is restricted, in low frequencies, to *Bos indicus* cattle. If, however, the D variant is suspected, alkaline-gel electrophoresis must supplement low pH runs. The same reasoning applies to examination of the purity of isolated casein components. Arave (2) has applied starch-gel electrophoresis at low

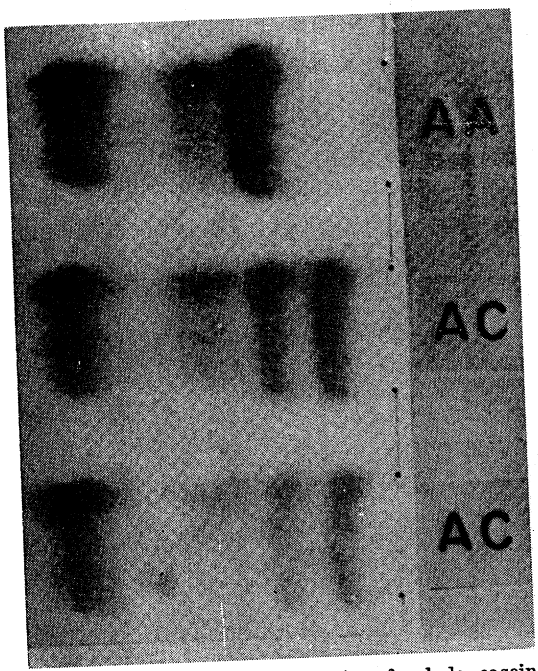

FIG. 6. Paper electrophoresis of whole casein at pH 7.15, 6.0 M urea. Electropherogram supplied by Dr. R. Aschaffenburg, Oxford, England.

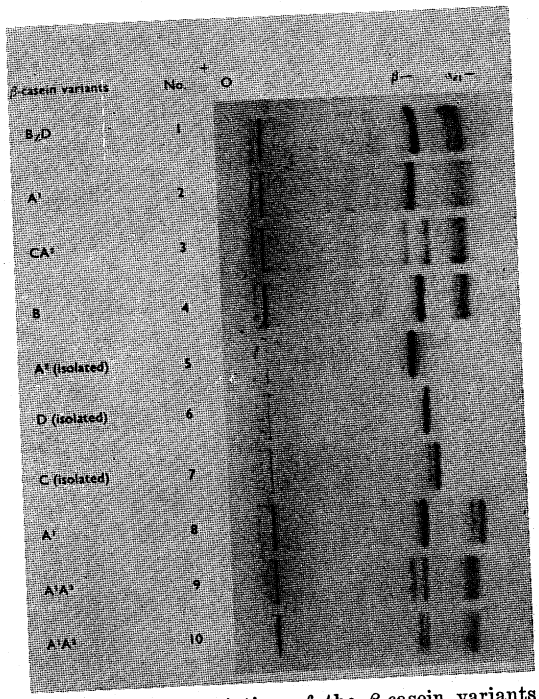

FIG. 7. Differentiation of the β-casein variants in acid polyacrylamide gel. O = origin. Numbers 1, 5, 6, and 10 are from Zebu cows, the others from Western cows. Reproduced with permission from Comp. Biochem. Physiol., 25: 177. 1968. (Fig. 3.)

pH to the resolution of β-caseins as illustrated in Figure 8.

All of the known variants of the α_{s1}-series (A, B, C, and D) and κ-caseins (A and B) can be detected by alkaline-gel electrophoresis alone (SGE or PAE) (25, 28, 32). Figures 4 and 9 illustrate the ease with which these variants are resolved. The κ-casein variants can be resolved only when mercaptoethanol (ME) is incorporated into either the protein solution or gel; otherwise an ill-defined smear results. With PAE it is recommended that ME be added to the protein solution and not to the gel itself, since ME often produces an undesirable (soft) gel. Either SGE or PAE (Fig. 4 and 9) serves to adequately resolve α_{s1}- and κ- variants.

The writer regards the phenotyping of γ-casein, and related components, as a special situation, and the reader is referred to the comprehensive review by Groves (18) for a thorough explanation of the complexity of these components.

The methods employed for the simultaneous phenotyping of milk proteins (2, 5, 10, 21) have all been performed on SGE; they materially simplify the problem of phenotyping the caseins and whey proteins, in that whole milk may be directly added to the gel and the saving in time is substantial. The only limitations of the method are as mentioned above, that β-caseins A^1, A^2, and A^3 cannot be resolved, and α-La migrates with α_{s1}-casein. The writer has attempted without success to employ PAE in simultaneous phenotyping of milk proteins—β-Lg B cannot be resolved from β-Cn-A.

Relative mobility measurements of caseins. When Wake and Baldwin (33) applied SGE to resolution of casein components they wisely suggested that the components be assigned a numerical value (Rm) which reflects the distance a component migrated relative to a particular component which was assigned a value of 1.00. Obviously, any component which migrates slower than the component designated 1.00 has an Rm less than 1.00. The component designated 1.00 (Fig. 10) appeared to be contained in all whole casein preparations and migrated close to the α_{s1}-casein region (1). Using this scheme, β-casein A had an Rm of 0.80 whereas α_{s1}-B had an Rm of 1.10.

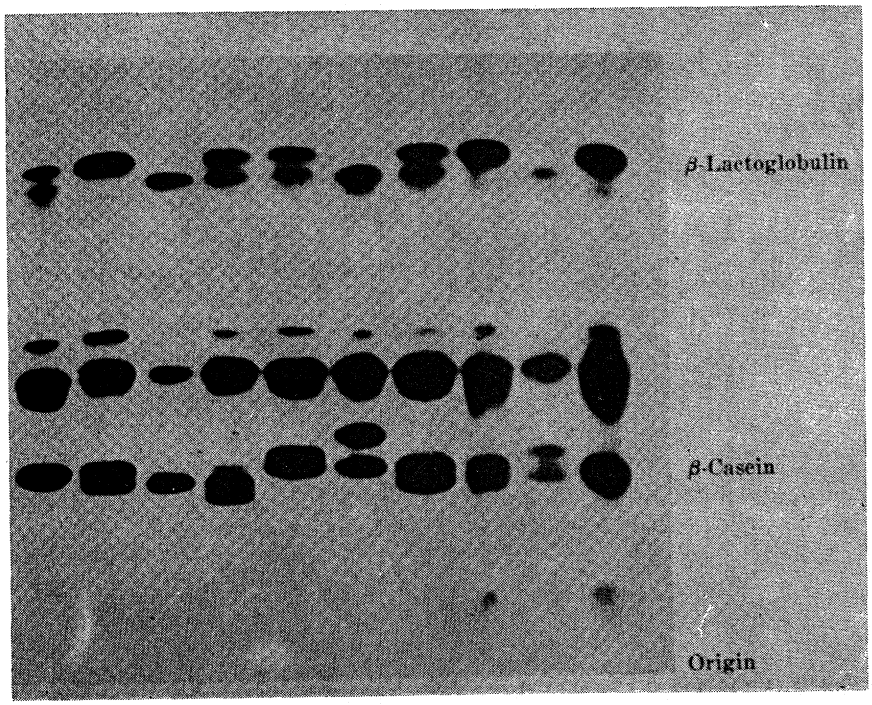

FIG. 8. Electropherogram of β-casein and β-lactoglobulin resulting from electrophoresis at low pH. Reproduced with permission from J. Dairy Sci., 50: 1321. 1967. (Fig. 1.)

Sample Number	1	2	3	4	5	6	7	8	9	10
β-Lg Phenotype	AB	A	B	AB	AB	B	AB	A	B	A
β-Cn Phenotype	A^1	A^1A^2	A^2	A^2A^3	A^1B	A^1C	A^1A^2	A^1A^2	A^2B	A^1

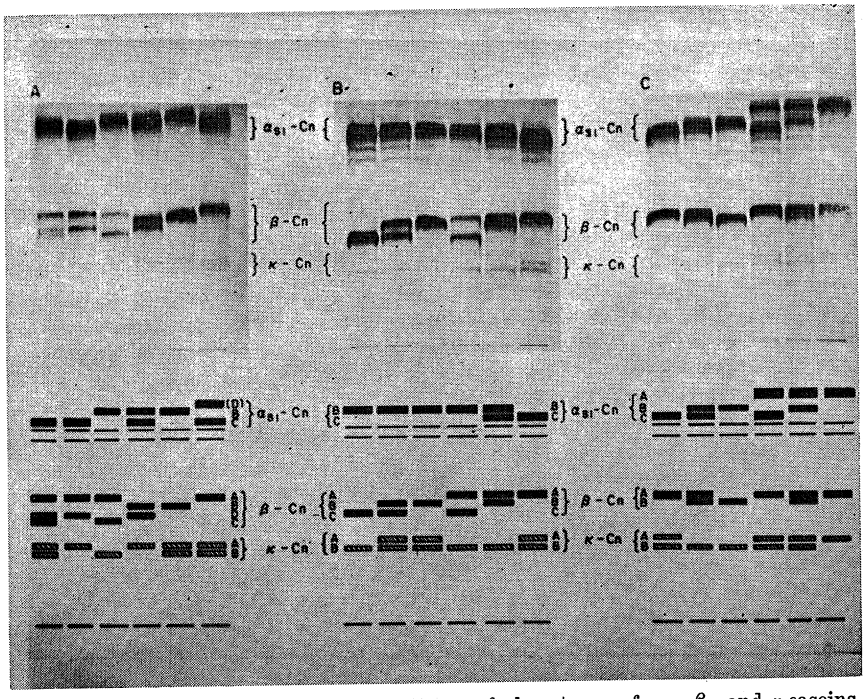

FIG. 9. Polyacrylamide gel electrophoretic patterns of phenotypes of α_{s1}-, β-, and κ-caseins. Gels run at pH 9.1, 4.5 M urea (mercaptoethanol added to samples). Thompson, unpublished.

The usefulness of Rm values becomes evident when genetic variants are being determined. But Rm values can be used effectively only when the methods (gel, buffer, etc.) are clearly defined and when the zone used for reference measurements is present and not genetically variable. Reference zone 1.00 is generally adequate since it is in practically all milks; Michalak (21), however, has shown that in a few instances (Fig. 11) Zones 1.00 and 1.04 were absent in the caseins studied. He also referred to the fact that Zones 1.00 and 1.04 have been genetically variable in the caseins of zebu cattle (*Bos indicus*). A reference sample of whole casein containing Zones 1.00 and 1.04 should be used in these two unusual instances.

The committee on Nomenclature and Methodology of Milk Proteins of the American Dairy

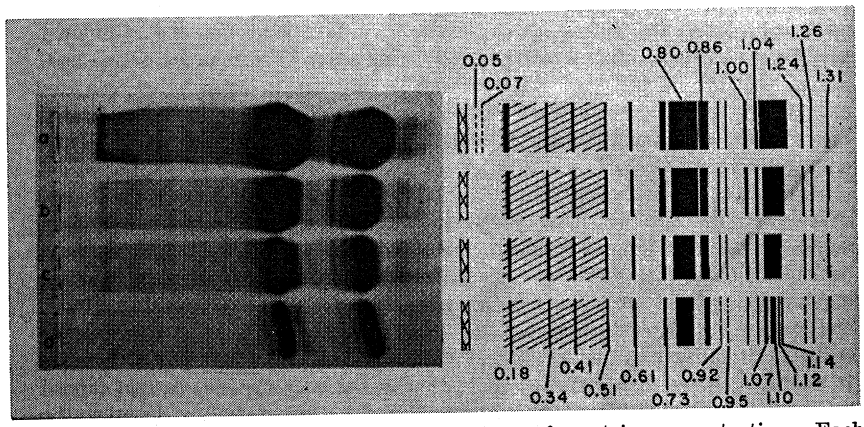

FIG. 10. Starch-gel analysis of whole casein: the effect of protein concentration. Each band is numbered by the distance it has moved from the starting slot, relative to band 1.00. a, 1.5% acid casein I; b, 0.8%; c, 0.5%; d, 0.25%. Reproduced with permission from Biochim. Biophys. Acta, 47: 225. 1961. (Fig. 1.)

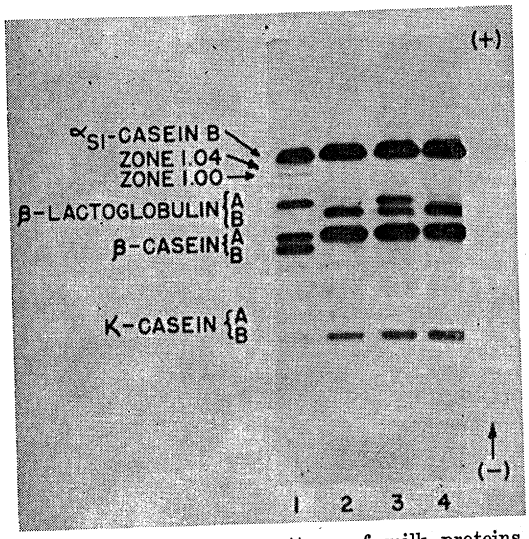

FIG. 11. Anomalous pattern of milk proteins. Number 1 is normal milk. Numbers 2, 3, and 4 have no bands of 1.00 and 1.04. Reproduced with permission from J. Dairy Sci., 50: 1319. 1967. (Fig. 1.)

Science Association (31) recognized that a zone numbering system would be appropriately applied when genetic variants were under consideration, and when newly discovered variants were being included in the scheme of nomenclature. For example, the discovery of α_{s1}-D (Rm—1.15) which migrates faster than α_{s1}-B (Rm—1.21) conveniently fits in the scheme (17). Likewise, β-casein D (Rm—0.57), which migrates faster than β-C (Rm—0.54) but slower than β-B (Rm—0.61), was easily accommodated into the nomenclature system (11). A summary of Rm measurements of known casein polymorphs appears in Table 1. They should be regarded as arbitrary measurements (unless identical electrophoresis methods are used) because they are influenced by buffers, pH, gel strength, etc.

TABLE 1. Relative mobilities (Rm) of α_{s1}- and β-casein variants by starch-gel and polyacrylamide-gel electrophoresis.

Variant	SGE (27, 30)	PAE (27, 30)
α_{s1}-A	1.18	1.22
D	?	1.15
B	1.10	1.13–1.14
C	1.07	1.10
(?)	1.04	~1.03
Reference zone[a]	1.00	1.00
(?)	0.86	~0.72
β-A[b]	0.80	0.65
B	0.76	0.61
D	?	0.57
C	0.70	0.54

[a] Using the zone reference system of Wake and Baldwin (33).

[b] β-Caseins A^1, A^2, and A^3 have the same Rm values at alkaline pH's.

The system of using Rm values for the identification of casein components has been successfully applied by Thompson and Kiddy (27), Thompson and Pepper (30), Grosclaude et al. (17), and El-Negoumy (15).

General Considerations

The foregoing discussion has involved those methods of electrophoresis which detect genetic variants that differ in net charge. Variants which do not differ in net charge offer special cases [such as β-casein β_Z (11, 26)]; amino acid composition and peptide finger-printing must supplement electrophoretic methods.

No one electrophoretic method can fully resolve the spectrum of milk protein variants; thus, both acid and alkaline-gel electrophoretic methods must complement each other.

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